The Effect of Collagen-Chitosan-Natrium Hyaluronate Composite on Expression of Vascular Endothelial Growth Factor (Vegf) Protein as Angiogenesis Reaction in Rabbit Corneal Stroma Wound (Experimental Study on Oryctolagus Cuniculus)

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Abstract

Background: Corneal wound healing involves several biological processes, various cytokines and growth factors are released in reaction to tissue damage, including Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), Platelet-Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF). VEGF is proinflammatory cytokines that increase microvascular permeability and plays an essential role in forming new blood vessels in the wound healing process. New blood vessels formed during the healing process can turn into pathological blood vessels, causing metabolic disorders in the cornea, resulting in corneal clouding and blindness. Keratoplasty is the primary therapeutic modality to replace pathological corneal tissue. Due to the limited availability of donors, many alternative therapies have been developed to restore corneal regeneration, one of which is using biomaterials in prosthetic devices with composite materials. Collagen-chitosan-natrium hyaluronate composite is a natural biopolymer considered to have good biocompatibility, biodegradable, and non-toxic properties. Besides being easy to get and produce, the composite is safe and improves corneal tissue regeneration.

Methods: Twenty adults New Zealand male white rabbits (30 eyes) were divided into three groups. The first group is the negative control group, without any treatment. The positive control group was the rabbits who had injuries to the stromal layer of the cornea, and the third group was the implant group, which were rabbits who received collagen-chitosan-natrium hyaluronate composite implants after their corneal stromal was injured. On day 14, rabbits were enucleated, and the corneal tissue was examined for immunohistochemistry using anti-VEGF antibodies.

Results: The study results showed a significant difference in the percentage of VEGF expression in the positive control group with the implant group was significantly different ($p = 0.000$, $\alpha > 0.05$). High VEGF protein expression did not exceed the positive control group indicating that the wound healing process is ongoing.

Conclusion: There was a significant difference in the level of VEGF protein expression between negative control group, positive control group, and implant group. The VEGF level in the implant group was lower than in the positive control group.

Keywords: cornea; biomaterials; composites; collagen; chitosan; natrium hyaluronate; VEGF; wound healing.

1. Introduction

The cornea is the outermost layer of the eye and, together with the eyelid and sclera, protects the eye. It is elastic, transparent, avascular, and has a thickness of about 500-600 micrometres. Corneal tissue has an angiogenic privilege, which is the ability of the cornea to maintain avascular condition by preventing neovascularization that grows from the surrounding tissue. Injury to the cornea can affect this condition by involving several biological processes, various cytokines and growth factors as a reaction to tissue damage, including Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), Platelet-Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF). VEGF proinflammatory cytokines play an essential role in forming new blood vessels called the angiogenesis process. However, the pathological new vessel can cause persistent inflammation, oedema, lipid deposition and corneal fibrosis, causing irreversible corneal damage and decreased visual acuity (Inomata *et al*., 2017;

Feizi *et al*., 2017).

The primary management of irreversible corneal damage is keratoplasty, however, due to the small number of human corneal donors, encouraging tissue engineering research to develop artificial corneal tissue that can be transplanted and serve as a substitute or as a facilitator of normal healing without causing side effects.

The two main components of tissue engineering products are cells and carriers. Tissue engineering uses the carrier method to form a scaffold designed in two or three-dimensional shapes made of biodegradable materials, synthetic and natural, thus providing a friendly microenvironment for cells to grow, differentiate and carry out the metabolic activities. Composite supporting materials must be met with specific requirements, including mechanical strength, controllable biodegradability, high porosity and optimal pore size, and suitable for increasing cell attachment, proliferation and migration (Karageorgiou *et al*., 2005; Chua *et al*., 2003). In addition, these materials and their degradation products must be biocompatible, that is, non-toxic, non-immunogenic and non-carcinogenic. In corneal tissue, a good biomaterial composition can reduce the accumulation and replacement of extracellular matrix components in tissue injury, mediate immune responses, prevent neovascularization and reduce fibroblast deposition, thereby reducing the formation of scar tissue (Weichang *et al*., 2014; Wang, 2013)

Various corneal biomaterial composites have been developed using amniotic membranes, fibrin, chitosan, hydrogel collagen, natrium hyaluronate and other biomaterials. Natural biopolymers such as collagen, chitosan, and natrium hyaluronate have been widely used to heal the wound. They have good biocompatibility, biodegradable, and non-toxic properties.

Collagen is the most abundant protein in mammalian tissues and a significant component of the natural extracellular matrix. Chitosan is a polysaccharide chitin derivative compound, has a similar structure to glycosaminoglycans, has properties like human body tissues and can be broken down by enzymes in humans (Drury *et al*., 2003). Hyaluronic acid is the simplest glycosaminoglycan and is found in almost every mammalian tissue; it is the main polysaccharide of the extracellular matrix, located both on the surface and inside the cell. Hyaluronic acid is biocompatible, biodegradable, non-toxic and forms a hydrogel due to its ability to absorb water. Chitosan collagen biomaterial composites can create a more compatible environment for cells than collagen or chitosan only. The addition of natrium hyaluronate increases the transmittance and transparency of biomaterials (Martinez *et al*., 2015).

Widiyanti et al. in 2019, researching artificial corneas based on collagen - chitosan – natrium hyaluronate showed no changes in chemical interactions, indicating that the three ingredients were successfully made. The artificial cornea is hydrophilic because the degree of contact is below 90°, then the artificial cornea also has a water absorption rate of 90%. These properties support cellular interactions and provide hydration to maintain eye clarity and permeability. Therefore, collagen-chitosan-natrium hyaluronate composites can be an excellent choice for making engineered corneas. The collagen-chitosan-natrium hyaluronate biomaterial composite also has advantages over the amniotic membrane, that it is easier to fabricate, so it does not depend on donor availability.

2. Methods

The negative control group were rabbits that were not injured by their stroma. The positive control group were rabbits whose corneal stroma was injured. and the implant groups were those rabbits whose corneal stroma was injured and a composite disc inserted. The observation was carried out for 14 days, and then the eyeball was enucleated for IHC examination of VEGF protein expression in the corneal stroma.

2.1 Animal

This study sample was an adult male New Zealand rabbit species Oryctolagus cuniculus with inclusion criteria rabbits weighing between 2.5 - 3 kg and declared healthy by a veterinarian. The exclusion criteria are rabbits with eye disorders and diseases. Drop out criteria are rabbits that died and experienced illness in their eyes or other organs during the clinical trial evaluation.

Rabbits are placed in a cage with a dimension of 90 cm x 60 cm x 60 cm; each rabbit occupies one cage, comfortable room temperature, safe environment, adequate ventilation and good nutrition.

Ethical eligibility was obtained from the Research Ethics Commission of the Faculty of Veterinary Medicine, Animal Care and Use Committee (ACUC), Airlangga University, Surabaya.

2.2 Collagen-chitosan-natrium hyaluronates (Col-Chi-NaHa) membrane preparation

Chitosan (Molecular Weight: 500.000-600.000) is crafted from chitin thru a deacetylation process (85-90% acetylation). Collagen and chitosan had been dissolved in 0.001 N hydrochloric acid separately. Then, the collagen and chitosan had been poured right into a beaker and stirred with a homogenizer for 30 minutes. Natrium hyaluronate solution with a purity of 95% is dropped into the combination and blended for half-hour. HPMC was brought to the

collagen solution as a crosslinker in this study. The ratio of collagen and HMPC used was 1: 1 by weight. So that the overall concentration of collagen and chitosan does not change, HPMC is added without increasing the quantity of the solvent. The collagen brought through HPMC is then stirred using a magnetic stirrer for 12 hours, and the temperature is maintained at 40ºC. After the collagen solution was cross-linked, the following procedure turned into the addition of 0.6% w / v chitosan and NaHA solutions. The homogeneous solution turned into placed on a Perspex plate and heated for twenty-four hours at 35ºC to achieve a dry membrane. The dry membrane was then immersed in PBS. Before getting used in in vivo research, the dry membrane was thoroughly rinsed in PBS, organized through 75% ethanol for 30 minutes to be sterilized and rinsed in a sterile PBS buffer solution (Widiyanti *et al*., 2019; Widiyanti and Prastyani, 2020).

2.3 Intrastromal implantation

Rabbits were placed on a sterile operating table, anesthetized with 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine given intramuscularly. The eye to be tested was given 0.5% pantocaine topical anesthetic. The intrastromal bag was made through a lamellar incision using a cataract surgery knife, then a composite with a thickness of 0.2 mm and a diameter of 3 mm was inserted into the bag (Tang *et al.,* 2011).

2.4 Histopathology

On the fourteenth day, the rabbit eye was enucleated. Immunohistochemical staining was performed on rabbit corneal stroma tissue. The corneal tissue was fixed with formaldehyde then made paraffin blocks. Furthermore, incubation was carried out using anti-VEGF-A antibody primers, rabbit polyclonal antibody, with 1:50 dilution.

2.5 VEGF immunoreactivity grading

Assessment of VEGF expression was carried out semiquantitatively using the Allred Scoring System rule, which sums the cell proportion score with the intensity score. The score for the proportion of cells divided into six levels based on the percentage of cells that express antibodies, 0 for 0%, 1 for <1%, 2 for 1-10%, 3 for 11-33%, 4 for 34-66%, and a score 5 for ≥ 67%. The cytoplasmic staining intensity score shows the intensity of the staining expressed by the cells where the score is 0 for no visible staining, 1 for weak, 2 for moderate, and 3 for strong intensity. The total score is added, and a range is obtained from 0-8. The total score is added, and a range is obtained from 0-8 (Fedchenko and Reifenrath, 2014; Allred *et al*., 1998).

2.6 Statistics

Variables with an interval scale are analyzed using the normality test then continued with Anova if the distribution is normal or Kruskal-Wallis if the distribution is not normal.

3. Results

The Effect Of Collagen-Chitosan-Natrium Hyaluronates Intrastromal Implantation On VEGF expression

The research results on thirty rabbit eyes subjected to the immunohistochemical examination of VEGF protein expression were shown in Table 1, the mean VEGF protein expression in the negative control group was 2,30, the positive control was 5,90, and the implant group was 4,80.

Table 1. VEGF Protein Expression

Group	VEGF Protein Expression							
	N	Mean	SD	Median	IQD	Min.	Max.	*p*
Negative control	10	2,30	0,48	2,0	1,00	2,00	3,00	0.000
Positive control	10	5,90	0,74	6,0	1,25	5,00	7,00	0.036
Implant group	10	4,80	0,63	5,0	1,00	4,00	6,00	0.012

*(significance < 0,05); SD : Standard Deviation; IQD : *interquartile deviation*

The results of the Kruskal-Wallis test in Table 2 showed that the *p*-value was <0.05, so it could be concluded that there were significant differences in the expression of the VEGF protein in the corneal stroma between the groups

being compared.

Table 2. Kruskal-Wallis test

Statistic	VEGF Protein Expression
Kruskal-Wallis	23.771
Df	2
Asymp. Sig.	0, 000

*(significance < 0,05); SD : Standard Deviation

Immunohistochemistry stain for VEGF, as shown in figure 1, showed a lower level of VEGF protein expression in the group of rabbits that received the composite after injury to the corneal stroma.

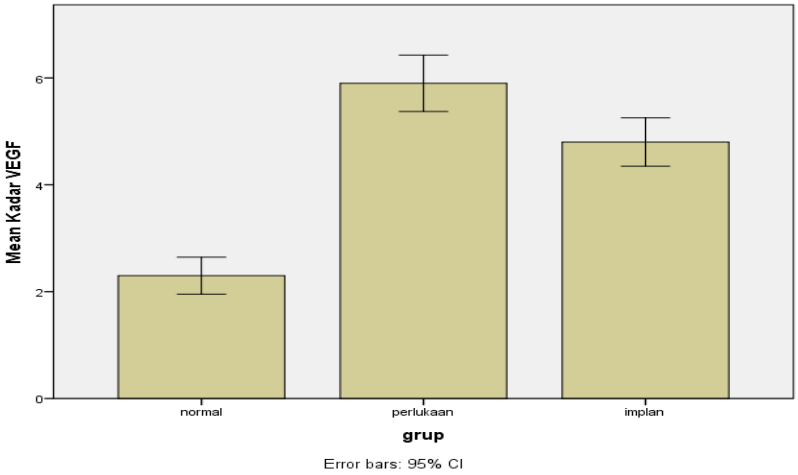

Fig. 1. Comparison of the mean VEGF protein expression between the negative control, positive control, and implant groups.

VEGF expression correlates with embryonic vascular growth, physiology, and pathological conditions in vivo. The level of VEGF expression used in this study was mouse-anti-VEGF with a 1:50 dilution. Positive values are shown in pale brown to dark brown staining, as shown in Figure 2.

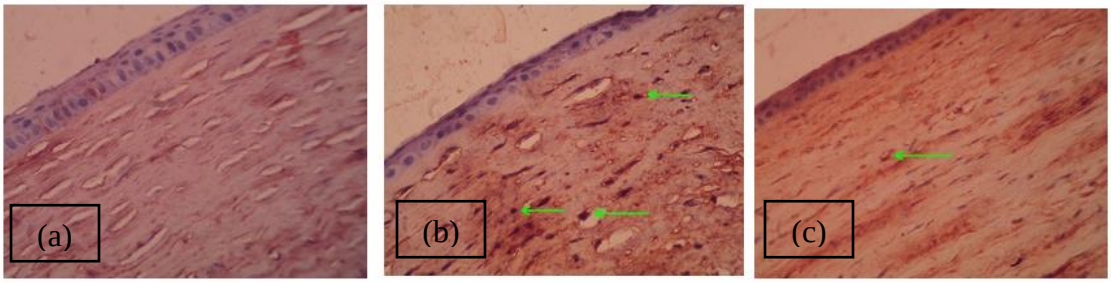

Fig. 2. The level of VEGF protein expression in the group (a)negative control, (b)positive control, and (c)implant group.

4. Discussion

The Effect Of Col-Chi-NaHa Intrastromal Implantation On VEGF expression

`The main proangiogenic growth factor is vascular endothelial growth factor (VEGF), produced in response to hypoxia and inflammation. VEGF expression is induced by inflammatory cells to reduce tissue hypoxia and metabolic deficiency through angiogenesis. VEGF activity peak occurs during the "window" period of 3 to 7 days after injury. After granulation occurs, angiogenesis stops, and blood vessels begin to regress, marked by apoptotic endothelial cells. Decreased VEGF and apoptosis loss can transition from hypercellular granulation tissue to hypocellular scar tissue (Stevenson *et al*., 2012).

This experimental research studies the VEGF protein expression and assessed it using the Allred Scoring System, which added the score of the proportion of cells with the intensity score. The results obtained were that there was a significant difference in VEGF expression in the implant group compared to negative and positive controls; according to research conducted by Stevenson et al., 2012 that VEGF began to decrease expression in 3–7 days after injury. Also, the VEGF level in the treatment group was lower than the positive control indicating that the VEGF that arose was a wound healing response, not a rejection reaction to the composite. This is following the research conducted by Hayashi et al. in 2016 observing the ability of the chimeric protein receptor VEGF 2 dissolved in corneal transplant model mice showed that controlling VEGF expression in mice given the chimeric protein receptor VEGF 2 resulted in significant suppression of the hemangiogenesis and lymphangiogenesis processes, thereby increasing the survival rate of the donor cornea.

Normal corneas in the negative control group showed VEGF expression, especially in epithelial cells, indicating that the angiogenic response depends on the balance of pro and antiangiogenic factor production. In contrast, in normal corneas, antiangiogenic factors function to block the angiogenic effects of VEGF. VEGF, typically produced by limbus epithelial cells in subangiogenic concentrations, plays an essential role in normal blood vessels' physiology and helps maintain vascular tissue (Wolfgang *et al*., 2000).

This means that the collagen-chitosan-natrium hyaluronate composite can prevent VEGF overexpression but is above the subangiogenic threshold to accelerate the wound healing process.

5. Limitation

1. This study has the limitation of making a stromal pocket not carried out with the guidance of optical coherence tomography, which has the advantage of increasing the accuracy of the pocket's location confined to the corneal stroma.

2. The study did not carry out serial observations. Suggestions for further research are necessary for serial observations (3, 7, 21, and 28 days) following the corneal wound healing timeline with a more extended study time. Observations in the timeline included the histology of leukocyte infiltration on day 3, fibroblast density on day 7, vascular endothelium on day 21, and collagen density on day 28.

6. Conclusion

Research on the effect of the collagen-chitosan-natrium hyaluronate composite on the expression of VEGF as an angiogenesis reaction in rabbit corneal stromal injuries can be concluded that collagen-chitosan-natrium hyaluronate composite resulted in a significantly lower VEGF expression compared to stromal injuries that were not treated with biomaterial composites.

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